Immunomagnetic Detection and Clinical Significance of Micrometastatic Tumor Cells in Malignant Melanoma Patients

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ABSTRACT

Purpose: Positive associations between the presence of micrometastatic tumor cells and disease aggressiveness have been reported in several tumor types, but the clinical implications are still not established. We wanted to test a new, sensitive immunomagnetic detection method on bone marrow (BM) and peripheral blood (PB) samples from patients with malignant melanoma and relate the findings to clinical outcome.

Experimental Design: Samples from 210 patients admitted for relapse of cutaneous melanoma were examined. Mononuclear cell fractions isolated from BM and PB were incubated with superparamagnetic particles coated with antimelanoma antibodies. Live tumor cells with bound beads were isolated with a magnet and identified in a microscope as cell-bead rosettes. Beads without antibody or with an irrelevant antibody were used as controls. The whole procedure was completed within 2–3 h. The identity of the cells was confirmed with a new double labeling procedure with fluorescent microspheres.

Results: Rosetted melanoma cells were found in BM aspirates of 35 of 186 (19%) patients, but in only 2 of 208 (1%) PB samples. The controls were all negative. After a median observation time of 1.1 year (range, 0–6.8 years), patients with tumor cells in BM showed a significantly shorter overall survival from time of BM aspiration (P = 0.009). In multiple regression analysis, a positive BM test was a strong indicator of overall survival (P = 0.021), associated with disease stage (American Joint Committee on Cancer) and with the number of metastatic sites, but not with the primary (Breslow) tumor depth and morphology.

Conclusions: The results demonstrate the prognostic significance of detecting BM micrometastasis in melanoma patients. The results strengthen the validity of the immunomagnetic technique. In contrast to other techniques, the method identifies intact, live tumor cells that can be further characterized, making the assay attractive for extended use.

INTRODUCTION

Malignant melanoma accounts for 1–3% of all malignancies (1) and has, in the last decade, been the most rapidly increasing type of cancer. The metastatic capacity of a melanoma is closely related to the vertical growth phase (2), and melanomas with a thickness of <0.75 mm have a remarkable cure rate of about 95% (3). The importance of this particular level may reflect that the basement membrane first becomes disrupted when the tumor is around 0.75-mm thick (4). Furthermore, tumors with a thickness of <0.75 mm are not vascularized (5, 6), making hematogenous spread of tumor cells from thin melanomas less likely. In contrast, 75–80% of patients with tumors exceeding 4 mm in thickness will die of their disease (3).

The fact that many radically operated melanoma patients later develop metastasis reflects that they had micrometastatic disease at the time of surgery. Although malignant melanomas may spread to virtually any organ or tissue, bone metastases are much less frequent than in most other types of cancer. However, such metastases may be difficult to detect by traditional methods because melanoma often appears mainly as small lytic foci (7) and hence may easily be overlooked. Immunocytochemistry studies in other types of malignancies, such as breast (8, 9), colon (10), lung (11), and gastric cancer (12, 13), have demonstrated the presence of tumor cells in bone marrow (BM) in a relatively high fraction of patients and provided evidence of the prognostic importance of such findings. The results in the published studies differ widely, which may be due in part to differences in the sensitivity and specificity of the methods used. The immunohistochemical methods have the advantage of permitting morphological evaluation of stained cells, but they are laborious, dependent on antibody specificity, and allow examination of limited numbers of cells. In melanoma, the occurrence of metastasis to BM, studied mainly by using reverse transcription-PCR (RT-PCR) techniques against tyrosinase in peripheral blood (PB) samples, is suggested to be relatively frequent (14–18). It should be noted, however, that nearly all RT-PCR studies have reported various methodological problems comprising contamination, transcription of pseudogene(s), or illegitimate transcription of the target gene (15–18). Immunomagnetic enrichment and detection (13) have the advantage of high sensitivity because more than 1 × 10⁷ mononuclear cells (MNCs) can easily be screened within less than 3 h, and after the
detection, the tumor cells are still alive and can be used for further characterization.

In the present study, we examined PB and BM samples from melanoma patients using a new assay with immunomagnetic beads to detect circulating tumor cells. The method was initially validated using cells from different melanoma cell lines added in vitro to normal MNCs, followed by immunomagnetic selection and microscopic detection of bead-binding cells. In the clinical study, the nature of the positively selected cells was confirmed by the binding of fluorochrome latex microparticles coated with an antibody recognizing another melanoma-associated antigen to the selected cells.

PATIENTS AND METHODS

Patients. Two hundred and ten patients previously operated on for histologically confirmed primary malignant melanoma were recruited when hospitalized on referral to our regional cancer center for follow-up therapy.

Sixty-four females (30%) and 146 male patients (70%) with a median age of 54.4 years were included. The majority of the patients received treatment with chemo- or radiotherapy after the sampling was done. One-hundred and two patients had American Joint Committee on Cancer (AJCC) melanoma stage 4, 87 patients had AJCC melanoma stage 3, 19 patients had AJCC melanoma stage 2, and 2 patients had AJCC melanoma stage 1. The criteria for inclusion were histologically confirmed primary malignant melanoma and no other known malignancy. The patients signed a written consent form. The study and the patient information form were approved by the regional Human Investigations Committee.

The median time from the excision of the primary melanoma to entry in the study was 2 years and 5 months (range, 0.03–18.3 years). The median observation time after the first sampling was 1.1 year (range, 0–6.8 years).

Antibody Conjugation to Beads. The monoclonal anti-melanoma antibody 9.2.27 (IgG2b isotype), kindly provided by Dr. Ralph Reisfeld (Scripps Institute, La Jolla, CA), binds to the core (250 kDa) of a chondroitin sulfate proteoglycan situated in the cell membrane (19). Experimental studies have shown that this antibody binds >90% of cells from different human melanoma cell lines (19, 20), but not to normal fibrous tissue, and shows no significant binding to normal BM cells (19–23). The IgG1 Ep-1 antibody recognizes 210-, 250-, and 400-kDa molecules in the melanoma cell membrane (24).

The MOC-31 antibody (IQ Products, Groningen, the Netherlands) was used as a negative control. MOC-31 recognizes the EP-CAM (EGP-2) antigen, which is consistently expressed in most epithelial cells, but not in normal hematopoietic cells or malignant melanoma cells (25). The 9.2.27 and MOC-31 antibodies were each bound to sheep antimouse IgG 4.5-μm superparamagnetic particles (Dynabeads SAM M450; Dynal, Oslo, Norway) by standard incubation procedure. The antibody concentration used was 2 μg/mg beads.

Processing of BM and PB Samples. Ten to 20 ml of BM were aspirated under local anesthesia from one side of the posterior iliac crest through aspiration needles (40-mm long and 2 mm in diameter) into syringes containing 1 ml of heparin (1000 IE/10 ml marrow). Forty ml of peripheral venous blood were drawn using heparinized vacutainers. Fat in the samples was separated by centrifugation (1000 × g for 5 min) and discarded. After 1:1 dilution with 1% PBS containing 1% FCS (Biochrom KG, Berlin, Germany), the samples were density centrifuged (1.077 g/ml Lymphoprep; Medinor, Oslo, Norway) at 1000 × g for 10 min. MNCs were collected from the interphase layer, washed in PBS with 1% FCS, and centrifuged (1000 × g for 5 min) before resuspension in 5 ml of PBS with 1% FCS. The MNCs were counted in an automatic counter (Cobas Micros OT; Roche Hematology, ABX-France, Montpellier, France), and the suspensions were diluted to contain 1 × 10^7 MNCs/ml and used thereafter in the detection assay.

The Immunomagnetic Detection Method. In parallel, 9.2.27 and MOC-31-coated and noncoated SAM IgG M450 beads were added to tubes containing the cell suspensions in a final volume of 1 ml with a ratio of beads to MNCs of 2:1. The cells were incubated with rotation at 4°C for 30 min, the suspension was diluted with cold PBS with 1% FCS, and the tubes were placed in a magnet holder for about 2 min. The supernatant was decanted off with the tubes still in the magnet holder. Fifteen-μl samples (of the positive fractions; remaining volume, 200 μl) were pipetted out for direct light microscopy to identify and count the number of rosetted cells, i.e., cells with membrane-bound beads. A schematic outline of the procedure is shown in Fig. 1A. A rosette was defined as a cell with >4 beads bound to its surface, evaluating the size, morphology, and three-dimensional picture as seen in the microscope. The finding of at least one rosette with 9.2.27-coated beads and no cells binding MOC-31-coated or SAM IgG beads was registered as a positive test (Fig. 1B). No cell binding was seen with any of these control beads. This cutoff level was set to avoid the risk of counting cells that are accidentally localized close to aggregates of a few beads as positive.

Sensitivity and Verification Studies. To determine the sensitivity of the method, we performed model experiments in which 20 melanoma cells from three different cell lines were prelabeled with a fluorescent stain (Acridine Orange) and, in different experiments, added to 2 × 10^6, 2 × 10^7, and 2 × 10^8 MNCs. Using the immunomagnetic method, the number of rosetted, fluorescence-stained tumor cells was determined in a light microscope equipped with an UV lamp.

In selected cases of clinical samples, the nature of the rosetted cells in the experiments was verified in double staining experiments. The Ep-1 antibody (a gift from Dr. P. G. Natali; Regina Elena Cancer Institute, Rome, Italy) was conjugated to 2-μm fluorescent latex microspheres ( Molecular Probes, Leiden, the Netherlands). Melanoma cells selected with 9.2.27 magnetic immunobeads from BM samples were incubated with such fluorescent beads, and the binding of both type of particles was examined using a Zeiss Axioskop (Carl Zeiss, Jena, Germany) equipped with appropriate illumination and filters.

Statistical Analysis. The association of immunomagnetic detection results and melanoma stage was explored by the χ^2 test for trend. Survival was measured from BM aspiration to death or date of latest observation. Patients still alive were treated as censored. Survival curves were estimated with the Kaplan-Meier method and compared by the log-rank test. The Cox proportional hazards model was applied for multivariate

RESULTS

In the model experiments with cell lines, it was found that 100% of the bead-rosetting cells fluoresced (not shown), with no bead-cell rosettes among the unlabeled normal MNCs, confirming the specificity of the method. Moreover, the fraction of recovered tumor cells was in range of 80–100%, depending on the cell line used, but independent of the number of MNCs to which the 20 melanoma cells were added.

An approximate sensitivity of 1–2 tumor cells/10^7 normal MNCs was achieved (data not shown).

Both BM and PB specimens were collected from 184 melanoma patients. BM specimens only were collected from 2 patients, and PB specimens only were collected from 24 patients (total, 210 patients).

Tumor cells (Fig. 1B) were detected in 19% (35 of 186) of the BM aspirates (Table 1). In some cases, five or more beads were observed associated with structures that could not be positively identified as cells. These cases were registered as negative.

The median number of rosetted cells in the positive samples was 3 (range, 1–1150). Of the positive BM samples, 30 had more than 1 cell detected, 28 had more than 2 cells detected, 10 had more than 4 cells detected, and 4 had more than 50 cells detected. This represents the number of rosetted cells found in one 15-μl fraction examined per sample. These numbers may represent approximately one-twelfth of the total number of immunobead-selected cells present in the entire sample.

In PB samples, only 2 of the 208 samples contained tumor cells. A BM aspirate was obtained from one of these patients and was positive. In none of the specimens examined were rosettes seen in the negative controls using MOC-31-conjugated and uncoated SAM M450 beads.

Double labeling experiments with antibody-coated fluorescent microparticles were performed in 40 of the cases. Binding of EP-1-coated fluorescent (blue) particles to cells with rosettes of 9.2.27-coated magnetic beads could easily be visualized (Fig. 1C), further confirming the nature of the rosetted cells.

Notably, a tendency of an increasing fraction of positive BM aspirates with more advanced melanoma stage (AJCC) was seen. Thus, whereas the patients with stage 1 disease (2 patients) were immunomagnetically negative, 2 of 16 patients with stage 2 disease tested positive. Twelve of 83 patients (14.5%) with

Table 1  Frequency of cases with tumor cells in bone marrow of melanoma patients

<table>
<thead>
<tr>
<th>Bone marrow</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>151</td>
<td>35</td>
<td>186</td>
</tr>
<tr>
<td>Percentage of patients</td>
<td>81</td>
<td>19</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2  Relationship between the presence of tumor cells in bone marrow and disease stage (AJCC) in melanoma patients

<table>
<thead>
<tr>
<th>Melanoma stage (AJCC)</th>
<th>No. of cases</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
<th>Stage 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>85</td>
<td>2</td>
<td>16</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td>Immunomagnetic</td>
<td>0</td>
<td>2 (12%)</td>
<td>2 (14%)</td>
<td>12 (14%)</td>
<td>21 (25%)</td>
</tr>
</tbody>
</table>

AJCC, American Joint Committee on Cancer.

N = 186; antibody, 9.2.27 antimelanoma antibody. The association between AJCC melanoma stage and positive immunomagnetic test was P = 0.06 (χ² test for trend).
stage 3 disease tested positive, and 21 of 85 patients (24.7%) with stage 4 disease had immunomagnetically detectable tumor cells (Table 2). The association between AJCC melanoma stage and positive test was assessed using the \( \chi^2 \) test for trend, giving a value of \( P = 0.06 \), but it should be noted that the number of stage 1 and 2 patients was very low.

Interestingly, in one AJCC stage 4 patient, tumor cells were found in the BM aspirate 10 months before overt metastases were detected by conventional examination by X-ray, computed tomography, and ultrasound, suggesting a possible benefit in using the rosetting method for monitoring the development of recurrent disease. When survival from the time of BM aspiration of patients with (35 patients) and without (151 patients) micrometastases was calculated, it was found that the presence of melanoma cells was associated with a significantly shorter overall survival (log-rank test, \( P = 0.009 \); Fig. 2). The multivariate analysis demonstrated that positive findings in the rosetting assay were, together with the number of metastatic sites and disease stage, the most important predictor of survival, thus demonstrating the clinical potential of this assay.

**DISCUSSION**

An immunomagnetic method for detecting live micrometastatic tumor cells was used on BM aspirates from 186 malignant melanoma patients with relapsed disease. A total of 19% of the cases had detectable tumor cells in BM, with the fraction of positives apparently increasing with disease stage (\( P = 0.06 \)) up to 25% in stage 4 patients. A significant relation between micrometastatic disease and survival of the patients was documented. Moreover, in multivariate analysis, the presence of melanoma cells in BM was, together with the number of metastatic sites and melanoma stage, the most important parameter of survival, both from time of testing and from time of removal of the primary tumor. The study demonstrates the validity of the method and its potential usefulness in the clinical management of melanoma patients.

More than 25 years ago Einhorn et al. (26) found by cytological technique that BM aspirates from stage 4 melanoma patients contained tumor cells in 9% (24 of 254) of the cases. In the same study, 15 of 96 (15%) patients examined at autopsy had BM involvement. Similarly, Aranha et al. (27) reported that BM aspirates were positive in 2 of 14 cases with AJCC stage 3 melanoma.

Most recent studies on melanoma micrometastasis have been performed with RT-PCR techniques on samples of PB using tyrosinase mRNA as a marker. This approach seemingly has the potential for detection of melanoma cells with high sensitivity (14). Smith et al. (14) reported initially that their assay could detect 1 cell from a melanoma cell line present in 2 ml of normal blood, results that later were not confirmed. Mellado et al. (28) found positivity in 36% of stage 1 and 2 melanoma patients, in 45% of stage 3 melanoma patients, and in 94% of stage 4 melanoma patients. They showed a positive test to be an independent prognostic factor. Brossart et al. (29) tried to establish a PCR-based semiquantitative assessment of melanoma cells in blood by interpolating the amplified tyrosinase signal strength in patient samples to an equivalent tyrosinase signal of diluted SK-mel 28 cells. They found that the number of circulating tumor cells correlated with tumor burden. In patients with regression of melanoma metastases after immunotherapy, a decrease in the calculated number of tumor cells in PB was observed.

Hoon et al. (30) introduced a multimarker PCR assay to further improve the sensitivity and demonstrated that the use of four markers in the PCR assay was significantly better than that of tyrosinase alone. Using four markers, they found that the probability of relapse for high-risk patients (33 patients previously treated for lymph node metastases) within the next 6 months was 3.8 times higher in those with a positive test.

Despite the encouraging results, it is known that RT-PCR methods may suffer from the risk of both false positive and negative results (15–17). In a recent comparative analysis of tyrosinase mRNA RT-PCR studies, Glaser et al. (16) found that five studies demonstrated frequencies of micrometastasis in stage 3 melanoma patients ranging from 13% to 50%, and in three of the studies, 90% or more positives were reported in patients with stage 3 melanoma. These conflicting results emphasize the need for standardization and quality control of the tyrosinase RT-PCR protocols. A study by the immunotherapy subgroup of the European Organization for Research and Treatment of Cancer Melanoma Cooperative Group (31), including nine different European laboratories and using different protocols and enzymes, showed that five of these laboratories obtained acceptable specificity and a sensitivity in detecting signals corresponding to about 10 cells/10 ml whole blood. Four laboratories had results evaluated as unacceptable in terms of specificity and/or sensitivity (17). Moreover, it has been discussed whether such inconsistencies could be related to very low levels of contamination (32), to illegitimate transcription of the tyrosinase gene (33), or to transcription of a pseudotyrosinase gene (34). Notably, even without such problems, amplified signals cannot be used reliably to estimate the number.

**Fig. 2** Overall survival from time of bone marrow aspiration (n = 186). A total of 151 patients tested negative (dashed line), and 35 patients tested positive (solid line). Censored patients (vertical marks) included 47 patients in the immunomagnetically negative group and 7 patients in the immunomagnetically positive group. Log-rank test, \( P = 0.009 \).
of tumor cells because the target mRNA level differs between cells in different tumors and patients, including the risk of missing signals in patients with amelanotic melanoma. We have recently used real-time RT-PCR targeting tyrosinase on 9.2.27-bead selected cell fractions, and we found that although it seemed specific, it was still less sensitive than cell-bead rosette detection alone (data not shown).

Our finding of only 19% positive BM tests in advanced malignant melanoma patients may seem low, but the discrepancy in comparison with most RT-PCR data might be due to the possibility of RT-PCR overestimating the presence of micrometastatic cells. Because the immunomagnetic method is dependent on consistent expression of the antigen recognized by the antibody used, the possibility cannot be ruled out that our assay did underestimate the frequency of micrometastasis. However, the ability of the 9.2.27 antibody to bind melanoma cells is well established (19–23). Furthermore, in spiking experiments, the minimum detection level was 1 tumor cell in $1 \times 10^7$ normal cells, a sensitivity level that also held up when $1 \times 10^8$ MNCs were screened (data not shown).

In our study, a very low fraction of the patients who tested positive in the BM sample also tested positive in the PB sample. It might be speculated that the BM in this context acts as a filter, whereas the PB is just a medium for transportation. It is noteworthy that in ongoing studies on samples from patients with uveal melanoma, a similar difference between blood and BM is seen, whereas in lung cancer and osteosarcoma patients, the discrepancy between the fraction of positives in blood and BM was much less pronounced.1

It might be argued that the rosetted cells might also include rare BM cells or even cutaneous cells contaminating the sample. Several additional lines of evidence argue against this possibility. It has been demonstrated that the antimelanoma antibody 9.2.27 has no significant binding to normal BM cells (19).

In vitro studies with fluorescence-labeled melanoma cells admixed with BM aspirates and PB samples showed that the cells isolated by the immunomagnetic method were the same melanoma cells as those added to the suspension. Importantly, BM from non-melanoma patients has consistently tested negative with 9.2.27 antibody-coated beads, and recently, we tested BM from 29 healthy volunteers, and all tested negative (data not shown). Furthermore, in the clinical cases in which another antimelanoma antibody conjugated to fluorescent particles was used in parallel, the evidence for the 9.2.27 bead-rosetting cells being melanoma cells was confirmed. Finally, it has also, in some cases, been possible to culture isolated cells from clinical samples (data not shown).

Multiple Cox regression analysis using survival from time of primary excision showed that positive BM test was an independent and more important predictor of overall survival than primary tumor depth. Tumor depth measured according to Breslow at the time of primary excision is a well-known and very good prognostic indicator when the disease is limited to the local or regional stage, but in advanced disease, the predictive value is fading. In our study, which included mainly AJCC stage 3 and 4 melanoma patients, multiple regression analysis showed that the Breslow tumor depth was not an important risk factor at this stage.

One major advantage of the immunomagnetic method used in this study is the simplicity and speed of processing, producing results in 2–3 h and allowing screening of as many as $10^7$ MNCs in a single procedure. Moreover, the isolated live cells are amenable for further immunological and molecular characterization. Other groups have reported on the use of either negative (35) or positive (36) immunomagnetic selection followed by immunocytochemical detection. However, with both approaches, the limitations associated with the immunostaining procedures remain, including that the tumor cells have to be fixed before the staining. In addition, positively selected cells with attached beads do not easily stick to the glass slide, resulting in the risk of losing target cells. Negative preselection is expensive because of the high number of beads required for pulling out the high number of bead-coated normal cells, and tumor cells are lost as a result of passive trapping of the cells. With our method, it is important to strictly follow the protocol and, in particular, to keep the cell suspension cold after the addition of magnetic beads to avoid nonspecific binding. Obviously, the properties of the targeting antibody are crucial. Using the MOC-31 anticarcinoma antibody in a study on colorectal cancer, a few false positive cells were observed in 3 of 206 BM samples from non-colorectal cancer patients (13), and in other studies with MOC-31-coated beads, a similar fraction of false positives has been seen.2 It is possible that a few immature cells in BM in some instances may express the target antigen, and work is initiated to identify these cells. However, the very low fraction of false positives seen with our method applied to other cancers does not represent a significant problem compared with its advantages relative to other methods.

It is concluded that the immunomagnetic method applied to BM samples may be used to identify melanoma patients with particularly poor prognosis and may possibly be used to select patients to receive more aggressive follow-up treatment. Finally, preliminary results (data not shown) indicate that the method can be useful in detecting early effects of treatment, i.e., as a surrogate marker for monitoring response to therapy.

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2 P. F. Brunsvig, S. Amdal, H. Hoifødt, and Ø. Fodstad, manuscript in preparation.
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